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Assessment of Neutralising Activity of Colostrum-Derived, Polyclonal, Bovine Antibodies: Use of the J774A.1 Anthrax Lethal Toxin Cytotoxicity Assay

Alison K. Pickering and Malcolm R. Alderton

Human Protection & Performance Division
Defence Science and Technology Organisation

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ABSTRACT

Bacillus anthracis lethal toxin is believed to play an important role in anthrax pathogenesis. Published reports have suggested that antibody therapies with specificity against lethal toxin may improve survival in animal models. Macrophage cell lines such as J774A.1 provide fast, convenient models to measure LeTx activity. Here, we report the use of the J774A.1 cytotoxicity assay to assess the neutralising activity of colostrum-derived, polyclonal, bovine antibodies. Antibodies against lethal factor and protective antigen were found to protect macrophages from lethal toxin, and should be investigated further as a treatment for anthrax.

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Telephone: (03) 9626 7000
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Executive Summary

Bacillus anthracis, the causative agent of anthrax, produces a toxin that is believed to play an important role in the pathogenic process of anthrax. The anthrax lethal toxin is comprised of two proteins, the protective antigen (PA) and lethal factor (LF). The inhalational form of anthrax is an aggressive infection that has a mortality rate of greater than 50%. Patients can die within 3 days of exposure to spore aerosols. Following the inhalation of *B. anthracis* spores, infection spreads to the lymph nodes and then into the bloodstream, releasing large quantities of the lethal toxin, which leads rapidly to death.

Recent animal studies have shown that the early administration of antibodies may provide protection against lethality following injection with lethal toxin or whole *B. anthracis* spores. By providing antibodies that neutralise toxin activity, the levels of toxin in the blood may be lessened or eliminated, thereby increasing survival. Such a treatment would require a readily available source of neutralising antibodies. Through collaboration with ANADIS Limited, DSTO has been able to access a source of colostrum-derived, *B. anthracis*-specific, polyclonal, bovine antibodies for testing and characterisation as a possible therapeutic treatment for anthrax. We discuss here, the assessment of these antibodies for neutralising activity using the J774A.1 macrophage cytotoxicity assay.

Through the use of the J774A.1 macrophage cytotoxicity assay, we found these antibody preparations to exhibit neutralising activity when present at equivalent concentrations, protecting macrophages from cell lysis. The demonstration of *in vitro* neutralising activity, suggests that colostrum-derived antibodies show early promise as a potential therapeutic treatment for human anthrax.

Authors

Alison Pickering Ph.D
Human Protection & Performance Division

Alison Pickering graduated with a Bachelor of Science with first class honours from James Cook University in 1996. The subject of her honours research was the development of an animal model for the potential BW agent, Burkholderia pseudomallei. A recipient of an Australian Postdoctoral Award, she chose to study the pathogenesis of Helicobacter pylori as the focus of PhD studies. Postdoctoral studies followed in the United Kingdom and in the United States of America, where she investigated the immune response to Bacillus anthracis and developed an animal model to study inhalational anthrax while working for the U. S. Food and Drug Administration. Alison joined HPPD in 2004 and has worked primarily on the development of rapid detection technologies for biological warfare agents.

Malcolm R. Alderton PhD
Human Protection & Performance Division

Malcolm (Mick) Alderton graduated from Monash University with a B Sc (Hons) in 1980 and a PhD (Immunology) in 1984. In 1994 he graduated with a Grad Dip Ed from RMIT and in 2000 he graduated with a Grad Cert Man from Flinders University.

In his first postdoctoral position with Biotechnology Australia he worked on the coccidiosis project at CSIRO Animal Health (Parkville). He moved to RMIT University in 1988 and worked as a Research Scientist and Lecturer before joining DSTO in 1995 as a Research Scientist. In January 1997 he was promoted to Senior Research Scientist and in 1998 became a Principal Investigator in collaboration with the Australian Membrane and Biotechnology Research Institute (AMBRI) Pty Ltd to develop ion channel switch (ICS) biosensors for defence applications. During 2001-2002, he undertook a 12-month sabbatical with the CSIRO Health Sciences and Nutrition Division. In July 2000 he became the Manager for the Novel Countermeasures for BW Agents Task. Currently he manages the Civilian Counter Terrorism on CBR Task. He is also the Program Manager for AAMOST Topic 126, the DSTO representative on the International Consequences Management Group, an Equity and Diversity Adviser, and the convenor of colloquia for HPPD.

Contents

1. Introduction

In animals, anthrax is a disease of herbivores, principally occurring in sheep, goats and cattle. *B. anthracis* spores are commonly found in the soil in farming areas in many parts of the world. Spores are inhaled or consumed by animals as they graze, rapidly causing a fatal illness. As the carcass decomposes, millions of spores contaminate the animal's remains. In humans, anthrax is almost always acquired through occupational contact with contaminated animal hides or carcasses. The most common form of human anthrax, representing more than 95% of cases, typically results from a cutaneous infection. The appearance of a characteristic, black, painless lesion on the hands, neck or face is the hallmark of cutaneous anthrax. Cutaneous anthrax is easily treated with antibiotics and rarely progresses to a systemic infection. If *B. anthracis* spores are inhaled, however, the resulting infection is much more serious. [For review of anthrax see Dixon *et al.* 1999; Inglesby *et al.* 2002]

Inhalational anthrax has a mortality rate of greater than 50%, as the infection quickly progresses from the lung to a septicaemic form (Meselson *et al.* 1994; Grinberg *et al.* 2001; Jernigan *et al.* 2001). Initial symptoms are non-specific, consisting of fever, non-productive cough, myalgia and malaise. Inhalational anthrax has an estimated incubation period of 1-6 days, however, death can occur within 24 hours of the development of respiratory symptoms. Late stage symptoms include stridor, cyanosis, respiratory failure, hemorrhage and shock. Even if diagnosis can be made quickly, antibiotic treatment for inhalational anthrax is not always effective.

B. anthracis has long been regarded as the most likely agent to be used as a biological weapon. This reputation is due to a number of factors including the hardy properties of the spore, the virulence of the organism and the non-specific nature of inhalational anthrax symptoms. *B. anthracis* produces two toxins that are thought to play important roles in the pathogenesis of anthrax; the lethal toxin and the edema toxin. The anthrax lethal toxin is made up of two proteins; the lethal factor (LF) and the protective antigen (PA). Both components are encoded on the virulence plasmid pXO1. Lethal factor; the active part of the toxin, is a zinc metalloprotease, while PA enables translocation of the toxin across target cells membranes. Following the phagocytosis of inhaled *B. anthracis* spores, alveolar macrophages transport the infection from the lungs to the lymph nodes (Ross 1957; Shafa *et al.* 1966; Dixon *et al.* 2000; Guidi-Rontani *et al.* 1999). Intracellular germination occurs and the vegetative cell begins the expression of the toxin genes (Guidi-Rontani *et al.* 2002). Lethal toxin activity is believed to enable the vegetative cell to escape phagocytosis *in vivo*, leading to the dissemination of infection into the bloodstream. Once present in the blood, the vegetative cell is protected from further attack by the innate, cellular immune response by the expression of an anti-phagocytic capsule (Zwartouw and Smith 1956).

The anthrax lethal toxin, was first identified in the 1950s as a factor present in filter-sterilized plasma from *B. anthracis*-infected guinea pigs that caused lethality when injected into uninfected animals (Smith and Keppie 1954). The precise mechanisms involved in the way the lethal toxin contributes to death are not well understood. It has long been known that lethality in systemic anthrax can occur despite bacterial clearance using antibiotics (Keppie *et al.* 1955), indicating a role for the anthrax toxins in pathogenesis beyond macrophage lysis. Recent

evidence suggests that the lethal toxin may also inhibit pro-inflammatory cytokines by blocking signaling pathways (Duesbery *et al.* 1998; Pellizari *et al.* 1999; Agrawal *et al.* 2003). Studies investigating the physiological consequences of lethal toxin injection have indicated that the toxin may cause pleural effusion, edema and hypoxic organ damage (Ezzell *et al.* 1984; Moayeri *et al.* 2003) but that the mechanisms by which this process occurs are dissimilar to those seen in response to lipopolysaccharide (Moayeri *et al.* 2003; Cui *et al.* 2004).

As the name suggests, the protective antigen component of the lethal toxin is a highly immunogenic protein. In whole spore animal vaccines, anti-PA antibodies are the immunodominant response while the human vaccine is designed to utilize the strong protective response that is elicited in response to PA (Friedlander *et al.* 1999). In the absence of immunisation, the administration of toxin-specific antibodies designed to block lethal toxin activity may provide an effective treatment to improve outcomes in inhalational anthrax. Recent studies have shown that toxin-specific antibodies may protect animals from death following lethal toxin injection and most importantly, from live infection (Little *et al.* 1990; Little *et al.* 1997; Kobiler *et al.* 2002; Zhao *et al.* 2003; Karginov *et al.* 2003; Sawada-Hirai *et al.* 2004). Additional evidence exists that anti-PA antibodies may also enhance the phagocytic uptake of *B. anthracis* spores and inhibit spore germination (Welkos *et al.* 2001).

Potential treatments that target the lethal toxin may be assessed initially by measuring the ability to prevent macrophage lysis *in vitro*. The lethal toxin has long been known to cause lysis in macrophage-type cells (Friedlander *et al.* 1986) and macrophage cell lines have been utilized by numerous groups as standard bioassays for the study of LeTx activity (Quinn *et al.* 1991; Farchaus *et al.* 1998; Zhao *et al.* 2003; Mohamed *et al.* 2004). In this study, we utilized the J774A.1 cytotoxicity assay to assess the ability of colostrum-derived anti-PA and anti-LF polyclonal antibodies to neutralize LeTx and to protect macrophages from cell death.

2. Methods and Materials

2.1 Colostrum-derived polyclonal bovine antibodies

2.1.1 Immunisation methodology, collection and purification of antibodies

The preparation, purification and characterisation of antibodies were carried out by ANADIS Limited (Campbellfield, Victoria, Australia) under a collaborative agreement with DSTO. Cows were immunised with commercially-available veterinary Anthrax Spore Vaccine (Colorado Veterinary Products, Denver, Colorado) according to the manufacturer's instructions. Approximately 6 months later, polyclonal antibodies were isolated from pooled, first milking colostrum. Caseins were removed from colostrum by pH adjustment to 4.6 with acetate buffer. Colostrum whey was subsequently centrifuged, filtered through 0.45 μm filters, adjusted to pH 7.5 and dialysed against PBS.

Antibodies recognising protective antigen or lethal factor were purified using affinity columns using standard methods. Briefly, PA or LF was coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) at 3 mg/mL of hydrated gel. After

whey was applied to the PA/LF specific columns, bound antibodies were eluted using 50 nM citric acid (pH 2.5), before neutralisation with 1 mM Tris ptt 8.0.

2.2 J774A.1 Cytotoxicity Assay

2.2.1 Anthrax Lethal Toxin: Acquisition, Reconstitution and Storage of Anthrax Lethal Factor and Protective Antigen

Recombinant anthrax lethal factor (rLF, Mol.Wt, 90,000) and protective antigen (rPA Mol.Wt. 83,000) were purchased from List Biological Laboratories (Campbell, California USA) as 1 mg quantities. Toxin components are provided in buffer which, when reconstituted according to the manufacturer's instructions in 1 mL of sterile distilled water, achieve a final buffer concentration of 5 mM HEPES with 50 mM NaCl at pH 7.5. The resulting 1 mg/mL solutions were frozen and stored at -20 °C in 50 µL quantities for single use.

2.2.2 Acquisition, Storage and Maintenance of J774A.1 Murine Macrophage Cell Line

The J774A.1 mouse macrophage cell line (TIB-67) was purchased from the American Type Culture Collection (Manassas, Virginia, USA). Upon receipt, the frozen cell suspension was thawed and cultured according to the instructions recommended by the ATCC. Culture medium consisted of high glucose Dulbecco's Minimal Essential Medium (Cat. No. 11965) supplemented with 10% (v/v) foetal bovine serum (FBS, Australian, heat-inactivated) and 1 mM L-glutamine. All culture medium components were purchased from Invitrogen (GIBCO, Grand Island, NY). Cultures were maintained in 75 cm^2 tissue culture flasks in an atmosphere of 5% CO_2. For subculture, adherent cells were detached from culture vessel surfaces using a cell scraper. Subculture was typically performed every 2-3 days using a sub-cultivation ratio of 1:5. For storage of the J774A.1 cell line, cells from confluent 75 cm^2 flasks were resuspended at 1 x10^7 cells/mL in FBS containing 10% DMSO and were frozen by a gradual decrease in temperature down to -80 °C before storage in liquid nitrogen.

2.2.3 Exposure of J774A.1 Cell Culture to Anthrax Lethal Toxin

For LeTx experiments, J774A.1 cells were seeded at 5x10^5 cells/mL into 96 well plates (200 µL per well). Plates were then transferred to the incubator for 2-3 hours to allow cells to adhere.

For all experiments, single aliquots of rPA and rLF were diluted to desired concentrations in complete culture medium. Antibody preparations were also diluted to the required concentration in complete culture medium. Anthrax lethal toxin was prepared by pre-combining rPA and rLF at various concentrations in 24-well plates either in the presence or absence of antibody in order to permit antibody-binding prior to addition to cell culture. Medium was removed from 96-well plate cultures before toxin (+/-Ab) preparations were added to the cells in 200 µL volumes. Plates were incubated at 37 °C for 4 hours.

2.2.4 Assessment of Cell Viability using MTT Tetrazolium Dye Assay

Following incubation for 4 hours, toxin-containing medium was removed from 96-well plate cultures. Medium was replaced by the addition of 0.5 mg/mL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-disphenyl-2H-tetrazolium bromide (MTT, Sigma, St Louis, MO) dissolved in complete culture medium. Plates were again returned to the incubator for 1 hour for viable cells to convert MTT to the insoluble, purple-coloured formazan intracellular precipitate.

After incubation, medium containing MTT was removed and was replaced with 100% DMSO. Complete colour development was found to occur after 30 min at RT, at which time absorbance at 540 nm was read using a spectrophotometer. For all experiments, each toxin concentration (+/-antibody) was assessed using 6-8 replicate wells. Antibody preparations were assessed in 2-3 independent experiments conducted on different days.

2.2.4.1 Quantification of cell viability using standard curve

In order to provide a quantifiable measure of cell viability, a 96-well plate containing decreasing cell densities was prepared for each experiment. To achieve a standard curve, the highest cell density of $5x10^5$ cells/mL was diluted in serial two-fold steps in complete culture medium (200 µL:200 µL). The cell density plate was treated in an identical fashion to all other plates, except cells were incubated for 4 hours in complete medium only (no toxin or antibody added). A standard curve was generated for each experiment in which cell density was plotted against absorbance at 540 nm. The highest cell density of $5x10^5$ cells/mL was defined as equating to 100% cell viability. For each experiment, the linear relationship generated between the absorbance at 540 nm and cell density was used to calculate the percentage of cell viability for all sample absorbance values.

3. Results

3.1 Demonstration of Lethal Toxin Activity in J774A.1 Cytotoxicity Assay

Following a series of preliminary, optimization experiments (results not shown), ideal ranges of LF and PA concentrations were determined in order to reveal the dynamic window of toxic activity under the experimental conditions of the J774A.1 cytotoxicity assay. Using these concentrations, potential treatments could be tested for the ability to neutralize the lethal toxin with the greatest sensitivity.

In Figure 1 (a), increased LeTx activity was observed as lethal factor was titrated against a constant concentration of protective antigen (100 ng/mL). As lethal factor concentration was increased from 0 to 100 ng/mL a steady decrease in cell viability was evident. Similarly, the titration of protective antigen against a constant lethal factor concentration (100 ng/mL) also resulted in an increase in LeTx activity. When protective antigen concentration was increased, cell viability was found to decrease (Figure 1 (b)).

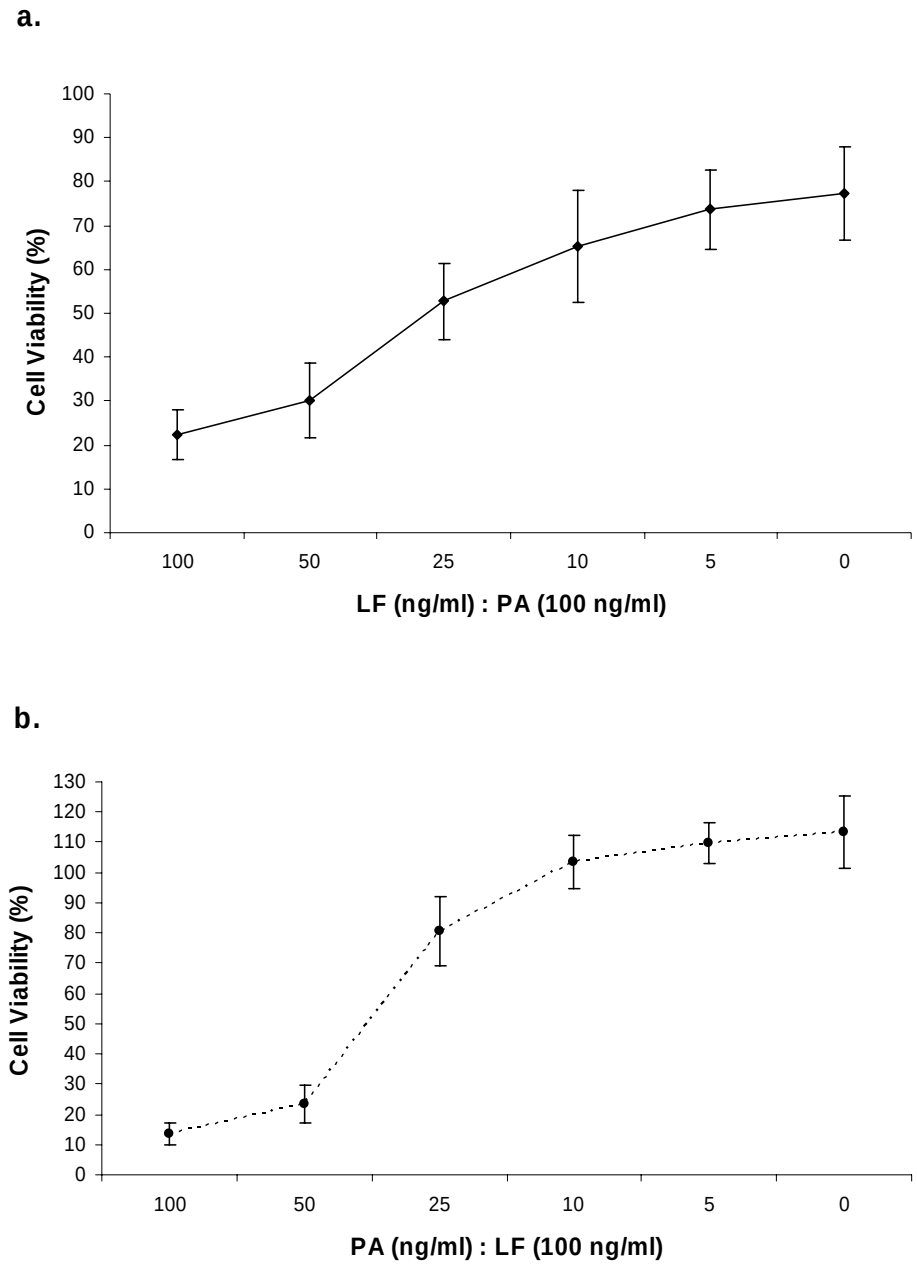

Figure 1. Anthrax lethal toxin activity as demonstrated in J774A.1 cell culture. a. Macrophages were exposed to various concentrations of lethal factor (100, 50, 25, 10, 5 and 0 ng/ml) titrated against a constant concentration of protective antigen (100 ng/ml). b. Macrophages were exposed to various concentrations of protective antigen (100, 50 25, 10, 5 and 0 ng/ml) titrated against a constant concentration of lethal factor (100 ng/ml). Four hours later, cell viability was assessed by incubation with MTT for 1 hour prior to development and spectrophotometric measurement at 540 nm. Cell viability (%) was quantified through the use of a cell density standard curve. Error bars represent 1 standard deviation of the mean of 6-8 wells.

3.2 Assessment of anti-PA Antibody Preparation in J774A.1 Cytotoxicity Assay

3.2.1 Assessment of neutralising activity

The potential neutralizing activity of the anti-PA colostrum-derived antibody preparation was assessed under the following conditions. Using a constant concentration of LF (100 ng/mL), the PA concentration was titrated from 0 to 100 ng/mL (1.2 nM). The anti-PA antibody preparation was assessed at the identical molar equivalent concentration (1.2 nM) and also at 1/5th that concentration (corresponding to approximately 0.25 nM). Treatment with both antibody concentrations was directly compared to J774A.1 cells exposed to toxin only (Figure 2).

When present at equivalent molar concentration (1.2 nM), the anti-PA antibody was found to neutralize target PA molecules and to increase cell viability. At PA 100 ng/mL (LeTx only), cell viability was measured at 23%. In comparison, the addition of anti-PA antibody (1x) resulted in 81% cell viability.

These results indicate that the anti-PA colostrum-derived antibody protects J774A.1 macrophages from lethal toxin activity when present at molar equivalent concentrations.

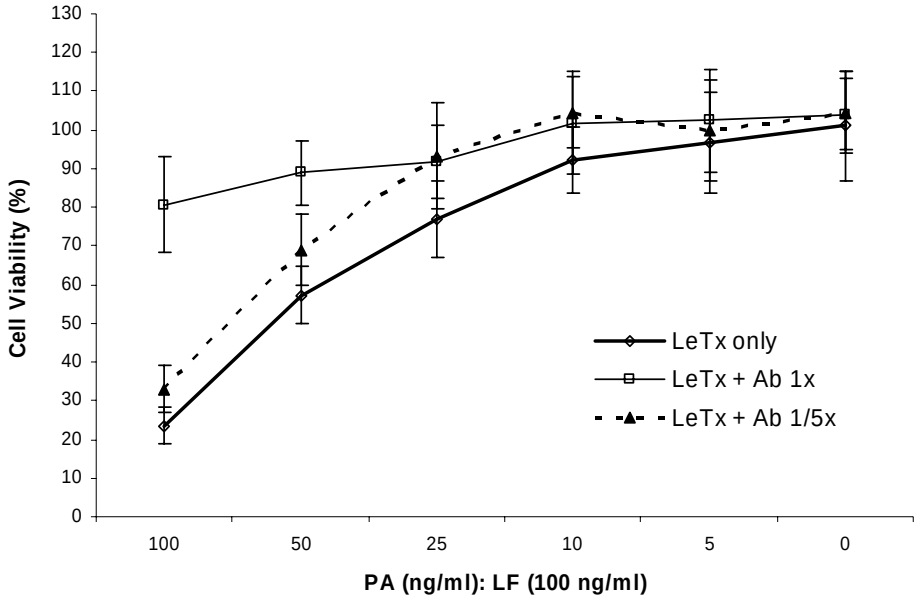

Figure 2. Assessment of neutralizing activity of anti-PA colostrum-derived antibody. J774A.1 macrophages were exposed to decreasing concentrations of PA in the presence of a constant concentration of LF. The highest PA concentration tested (100 ng/mL) corresponded to a molar concentration of 1.2 nM. Anti-PA antibody was tested at two different concentrations corresponding to 1x (molar equivalence, 1.2 nM) and 1/5x (0.25 nM) the concentration of the PA target protein. Error bars represent 1 standard deviation of the mean of 6-8 wells.

3.3 Assessment of anti-LF Antibody Preparation in J774A.1 Cytotoxicity Assay

3.3.1 Assessment of neutralising activity

The potential neutralizing activity of the anti-LF colostrum-derived antibody preparation was assessed under the following conditions. A constant concentration of PA (100 ng/mL) was added to all wells, while LF concentrations were decreased from 100 ng/mL (1.2 nM) to 0 ng/mL. Anti-LF antibody was assessed for neutralizing activity at the identical equivalent molar concentration (1.2 nM) and at 1/5th of that concentration (0.25 nM). Both antibody concentrations were directly compared to J774A.1 macrophages treated with toxin only (Figure 3).

When present at a molar equivalent concentration (1.2 nM), the anti-LF antibody was found to neutralize LeTx activity, protecting J774A.1 cells from toxin and preserving cell viability. When cells were exposed to LeTx only (LF 100 ng/mL), 15% viability was observed. In comparison, 71% of cells remained viable when anti-LF antibody was present in addition to LeTx. Some protection was also observed in anti-LF treated wells to which antibody was added at 0.25 nM. When anti-LF was present at 1:5 (LeTx : Ab), cell viability was also significantly increased (37%) in comparison to cells that were exposed to toxin only (15%).

These results indicate that anti-LF colostrum-derived antibody protects J774A.1 macrophages from lethal toxin activity at molar equivalent concentration. Some protection may even be provided when LF is present in excess to antibody.

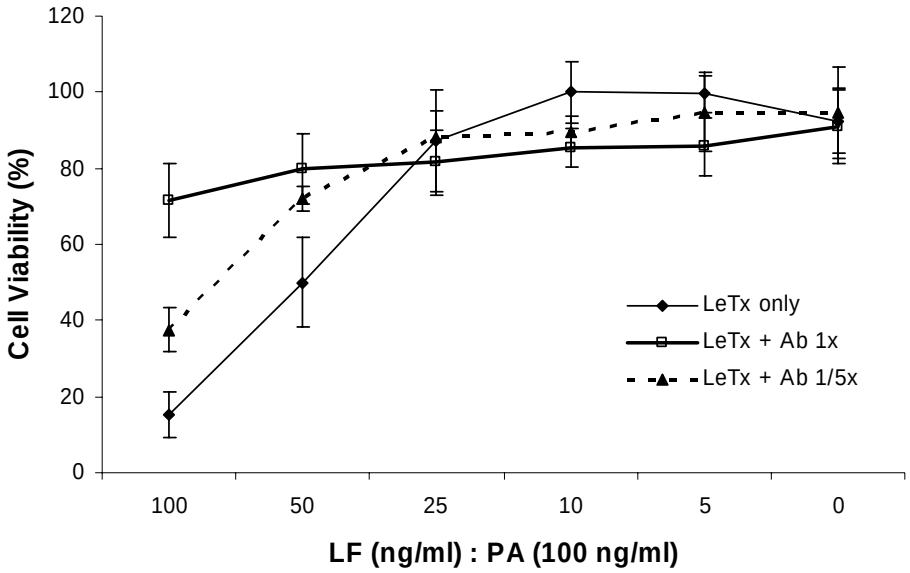

Figure 3. Assessment of neutralizing activity of anti-LF colostrum-derived antibody. J774A.1 macrophages were exposed to decreasing concentrations of LF in the presence of a constant concentration of PA. The highest LF concentration tested (100 ng/mL) corresponded to a molar concentration of 1.2 nM. Anti-LF antibody was tested at two different concentrations corresponding to 1x (molar equivalence, 1.2 nM) and 1/5x (0.25 nM) the concentration of the LF target protein. Error bars represent 1 standard deviation of the mean of 6-8 wells.

4. Discussion

Macrophage cell lines such as J774A.1 provide a fast and convenient cell culture model to study lethal toxin activity *in vitro*. We describe here, the use of J774A.1 macrophages to assess the potential neutralizing activity of PA and LF specific colostrum-derived, polyclonal, bovine antibodies. Using this cell culture model, cell viability following exposure to anthrax lethal toxin was quantified by MTT tetrazolium dye incorporation.

During preliminary optimization experiments, the ideal concentrations of the lethal toxin proteins, PA and LF were determined. By optimizing PA and LF concentrations to those within the dynamic range of the J774A.1 assay, we were able to examine the effect of antibody treatment with the maximum sensitivity. The concentrations of lethal factor and protective antigen used in these experiments were coincidently very similar to those previously described by Quinn and colleagues (1991) among others, demonstrating the consistency of the assay in different laboratories and the suitability of the J774A.1 cytotoxicity assay for use in assessing potential treatments designed to prevent anthrax lethal toxicity *in vitro*.

An exposure time of 4 hours was chosen arbitrarily for all experiments. Previous publications have all described an incubation time of 3-4 hours following addition of lethal toxin (Quinn *et al.* 1991; Farchaus *et al.* 1998; Gupta *et al.* 1998; Mohamed *et al.* 2004). Four hours, therefore, was assumed to provide sufficient time for toxic activity to occur before cell viability was assessed using the MTT tetrazolium dye assay, whilst also keeping the overall assay time to a minimum.

The results presented here indicate that colostrum-derived, polyclonal, bovine, anti-LeTx antibodies display neutralizing activity against anthrax lethal toxin, protecting macrophages from cytotoxic effects *in vitro*. When present in equimolar concentrations, anti-PA and anti-LF antibody preparations were able to provide significant protection to J774A.1 cells. The mechanism of protection may be presumed to occur through the inhibition of toxin assembly at the macrophage cell membrane.

The mechanisms by which lethal toxin contributes to death in inhalational anthrax are not well characterized. In addition to the ability to trigger cell lysis in macrophages, studies have indicated that anthrax lethal toxin may also exert an inhibitory effect on pro-inflammatory cytokine expression and may contribute to the development of pleural effusion and hypoxic organ damage, as demonstrated in animal models (Ezzell *et al.* 1984; Moayeri *et al.* 2003; Cui *et al.* 2004). Anti-PA antibodies have also been shown to influence phagocytosis and spore germination (Welkos *et al.* 2001). Treatment with LeTx-specific antibodies, therefore, has the potential to influence anthrax pathogenesis through a number of different mechanisms beyond the intended neutralization of lethal toxin activity.

As a potential treatment, specific antibodies show initial promise for the inhibition of the lethal toxin activity. To determine the efficacy of antibody treatment on *B. anthracis* infection, however, such activity needs to be tested *in vivo*, presumably using an animal model which incorporates a live *B. anthracis* spore infection, preferably delivered via the aerosol route.

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Alison K. Pickering and Malcolm R. Alderton

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Alison K. Pickering and Malcolm R. Alderton

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DSTO Defence Science and Technology Organisation
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Fishermans Bend Victoria 3207 Australia

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19. ABSTRACT

Bacillus anthracis lethal toxin is believed to play an important role in anthrax pathogenesis. Published reports have suggested that antibody therapies with specificity against lethal toxin may improve survival in animal models. Macrophage cell lines such as J774A.1 provide fast, convenient models to measure LeTx activity. Here, we report the use of the J774A.1 cytotoxicity assay to assess the neutralising activity of colostrum-derived, polyclonal, bovine antibodies. Antibodies against lethal factor and protective antigen were found to protect macrophages from lethal toxin, and should be investigated further as a treatment for anthrax.